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Bacterial expression and purification of biologically active mouse c-Fos proteins by selective codon optimization

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Abstract A simple strategy using selective codon optimization was devised to express mouse c-Fos protein in high levels in *E. coli*. Ten arginine codons located in the basic region were optimized to achieve high levels of protein expression. The c-Fos protein was purified to near homogeneity and was demonstrated to be biologically active by assaying its several biological activities.

Key words: High-level bacterial expression; Mouse c-Fos; Selective codon optimization

1. Introduction

Activator protein (AP-1) was originally identified as a transcriptional factor binding to a *cis*-element of the human metallothionein IIa (hMT IIa) promoter [1]. The binding site for AP-1 was very quickly recognized as the TPA (12-O-tetradecanoylphorbol 13-acetate) response element (TRE) of several cellular and viral genes including human MT IIa, collagenase, stromelysin, c-jun, interleukin 2 (IL2), SV40 and polyoma [1]. In addition to TPA, AP-1 activity has been shown to be regulated by many other stimuli including growth factors, cytokines and DNA damaging agents [1].

Subsequent studies revealed that AP-1 is a dimeric complex of the gene products of two different gene families: jun family and fos family. The jun family includes c-jun, JunB and JunD and fos family includes c-fos, fosB, fra1 and fra2. While different Jun proteins can homo- and heterodimerize within the family and bind TRE specifically, different Fos proteins cannot. However, Fos proteins can dimerize with Jun proteins and bind TRE specifically regular size[1].

AP-1 activity can be altered by regulating the level of different Jun and Fos proteins or alternatively by modifying different Jun and Fos proteins in response to extracellular signals. The easy availability of recombinant c-Jun proteins has been instrumental in determining its role in regulation of genes involved in many different biological processes and in elucidating the detailed signal transduction pathway regulating its activity in response to external stimuli.

It is well documented that synonymous codons are not used randomly in various organisms and these nonrandom usage of synonymous codons has been shown to correlate with the relative quantities of various tRNAs [2]. It has been suggested that the codon usage may affect protein expression in the cell [2]. This concept has been tested in a few studies [see [3] for an example]. These studies showed that insertion of rare codons into highly expressed cDNAs can negatively affect the expression level of the mutated cDNAs, and furthermore, the effects were much stronger when the inserted rare codons were located closer to the initiation codon. In fact, some groups have achieved high levels of expression of different genes by using total synthetic cDNAs based on the optimal codon usage concept [4–6].

Although rat c-Fos protein has been expressed in and purified from *E. coli* by using a totally synthetic c-fos cDNA with optimal *E. coli* codons [4], mouse and human c-Fos proteins have not been expressed in *E. coli*. The strategy used for expressing rat c-Fos in *E. coli* is theoretically applicable to the expression of other mammalian genes in *E. coli*, but the strategy has not often been adopted for practical reasons.

Here I describe the expression and purification of mouse c-Fos in *E. coli*. The strategy is simpler than the previous one used for expressing rat c-Fos proteins in *E. coli* and therefore should be more easily adaptable to the expression of other mammalian genes in *E. coli*.

2. Materials and methods

2.1. Construction of pTH6-cfos

pTD2 [7] was modified to generate pTH6, which contains a translation initiation codon followed by 6 histidine codons and then a multiple cloning region with *Nco*I, *Nhe*I, *Eco*RI, *Hin*dIII and *Bam*HI in the stated order. The detailed description of pTH6 construction will be available upon request.

To construct pTH6-cfos, a fragment between a *Sal*I in the 3′ untranslated region of mouse c-fos cDNA and a *Sal*I in the multiple cloning region of pGEM4-fos was deleted by digesting with *Sal*I and relegating. Then the *Eco*RI-*Hin*dIII fragment containing c-fos cDNA was subcloned into Bluescript KS(+)II and single-stranded DNA was obtained. The single-stranded DNA was used for mutagenesis to create a *Nco*I site at the translation initiation site. Mutagenesis was performed on the single-stranded template using the appropriate oligodeoxynucleotides (whose description is available upon request) and the Amersham site-directed mutagenesis kit as recommended by the manufacturer. The mutations were initially identified by creation of restriction sites. Final confirmation was by direct sequencing. The c-fos cDNA fragment containing the entire coding region was subcloned into pTH6 to generate pTH6-cfos.

2.2. Expression and purification of recombinant cFos proteins

To express c-Fos, pTH6-cfos plasmids containing wild-type and different codon-optimizing coding DNAs were transformed into *Escherichia coli* BL21 (DE3) pLysS. The cells were grown to an OD of 0.6 in a 37°C shaking incubator, induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Samples were taken at different times after induction and analyzed by SDS-PAGE. The plasmid expressing the highest level of c-Fos was used for expression and purification of c-Fos. The Fos proteins were purified from inclusion bodies and renatured as described [8]. Protein concentrations were determined by the Bradford assay (Bio-Rad).

2.3. Immunoblotting

The proteins were resolved by electrophoresis in a 10% SDS-PAGE

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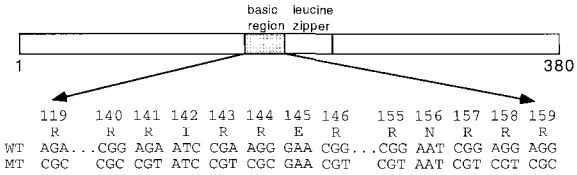

Fig. 1. A diagram of c-Fos and the codon optimization scheme. The basic region and the leucine zipper region of c-Fos is shown. The ten wild-type arginine codons located between amino acid 119 and 159 of the basic region and the optimized codons are shown. The mutant containing the optimized arginine codons from amino acid 140 to amino acid 146 is designated as M1 and the mutant containing all ten optimized codons M2.

and electro-transferred to a nitrocellulose. The nitrocellulose was blocked in PBS buffer (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5) with 0.1% Tween 20 supplemented with 5% nonfat milk at 4°C. Fos proteins were detected by incubation of the nitrocellulose with Fos antibody ^{14}Cl (1:1000) at 4°C for 1 h [9], followed by rinsing and detection with an Amersham ECL kit. The secondary antibody was also diluted 1:1000.

2.4. Mobility-shift assay

Mobility-shift assays contained the indicated amounts of the different cJun/JunB chimera CB4 [4] and Fos proteins, 1 ng of ^{32}P-labeled collagenase phorbol 12-myristate 13-acetate response element (TRE) probe, 5 μg poly(dI·dC), 12 mM HEPES·KOH (pH 8.0), 50 mM KCl, 6 mM $MgCl_2$, 1 mM EDTA, 10% (vol/vol) glycerol, 5 mM dithiothreitol, and 80 μg of bovine serum albumin in a total volume of 20 μl. After a 20-min incubation at room temperature, reaction mixtures were loaded on 5% native polyacrylamide gels (acrylamide/bisacrylamide, 40:1). Electrophoresis was done in 0.4×Tris/borate/EDTA buffer (TBE; 1×TBE is 90 mM Tris/90 mM boric acid/2 mM EDTA, pH 8.3) at room temperature. The gel was dried and exposed to an X-ray film.

2.5. Kinase reaction and two-dimensional phosphopeptide mapping

The recombinant c-Fos proteins were incubated at 30°C for 30 min with the relevant kinases in kinase buffer (20 mM HEPES, pH 7.3, 10 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenylphosphate, 0.1 mM $NaVO_4$, 2 mM DTT) containing 10 μM [γ-^{32}P] ATP in 20 μl. Reactions were terminated by addition of 6.5 μl 4×SDS-PAGE sample buffer and boiling. Purified ERK1/2 is a mixture of both enzymes (gift from M. Cobb) and PKA is from Promega. The reaction mixtures were loaded on a 10% SDS-PAGE gel. The gel was dried and exposed to an X-ray film (Amersham). For two-dimensional phosphopeptide mapping, the gel-resolved proteins were electrophoretically transferred to a nitrocellulose (Schleicher and Schuell). The nitrocellulose was exposed to an X-ray film. The phosphorylated c-Fos bands were excised and digested by trypsin (Worthington). The samples were further processed to obtain the two-dimensional peptide mapping as described [10,11].

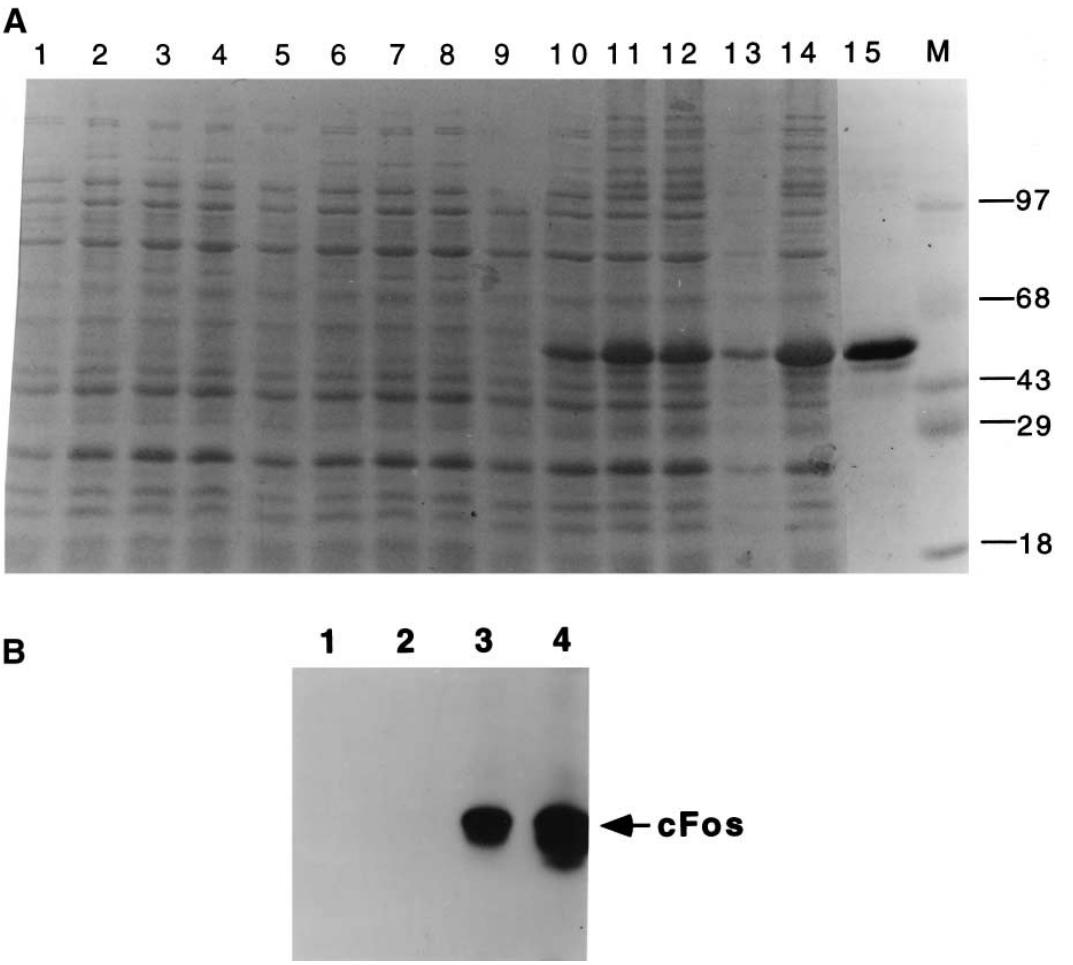

Fig. 2. Analysis of protein expression of different c-Fos expression vectors. (A) SDS-PAGE analysis of various bacterial samples. Lanes: 1–4, samples of pTH6-cFos at 0, 1, 3, 5 h after IPTG inductions; 5–8, samples of pTH6-cFos M1 at 0, 1, 3, 5 h after IPTG induction; 9–12, samples of pTH6-cFos M2 at 0, 1, 3, 5 h after IPTG induction; 13, soluble fraction of the 5 h sample of pTH6-cFos M2; 14, inclusion body fraction of the 5 h sample of pTH6-cFos M2; 15, purified c-Fos. Lane M, molecular weight markers. The size of each marker is indicated at the right and labeled in kDa. (B) Immunoblotting analysis. Lanes: 1–3, 3 h after IPTG induction samples of PTH6-cFos, M1 and M2, respectively; 4, purified cFos. The cFos position is indicated.

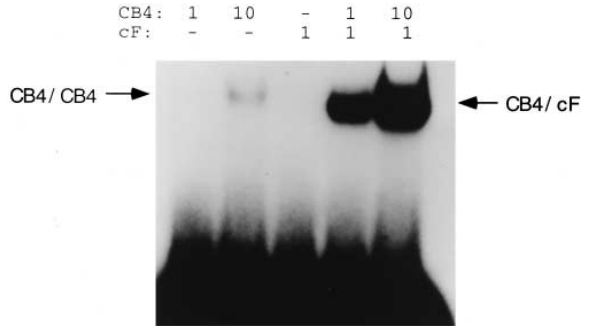

Fig. 3. Stimulations of CB4 DNA binding activity by c-Fos. The indicated amount of protein (ng/reaction) was mixed with a ^{32}P-labeled Col-TRE probe. The migration positions of the different protein-DNA complexes are indicated. The free probe is at the bottom.

3. Results

To express c-Fos in bacteria, wild-type c-fos cDNA was inserted into pTH6 and pET-8c, respectively. A schematic illustration of c-Fos is shown in Fig. 1. As shown in Fig. 2A, c-Fos proteins were not detectably expressed from the expression vector pTH6-cfos. The same results were also obtained with pET-8c-cfos (data not shown).

One possible explanation for the failure to express mouse c-Fos at high levels in *E. coli* is the prevalence of codons infrequently utilized in *E. coli* in the c-fos sequence [12]. Careful examination of mouse c-fos sequence revealed that the most significant difference of codon usage between the mouse c-fos and the genes highly expressed in *E. coli* is the codons for arginine. Therefore, attempts were made to optimize codon usage for arginine of mouse c-fos. It should be noted that codon optimization does not alter the encoded amino acid sequence. Initially, five arginine codons between amino acid 140 and 146 were optimized (Fig. 1). However, this optimization was still not enough to express c-Fos at high levels in *E. coli* (Fig. 2A). Additional five arginine codons, one for Arg 119 and the other four for arginine codons between amino acid 155 and 159, were optimized (Fig. 1). Significantly, high levels of c-Fos were expressed in *E. coli* (Fig. 2A). The highest level of expression was obtained 3 h after IPTG induction and was determined to be about 20% of total bacterial proteins based on density scanning quantitation of the Coomassie Blue-stained gel shown in Fig. 2A. c-Fos proteins were present in both the soluble fraction and the inclusion body. c-Fos proteins were purified from the inclusion body. The purity of the mouse c-Fos protein was estimated to be at least 95% based on density scanning quantitation of the Coomassie Blue-stained gel (Fig. 2A).

As shown in Fig. 2A, some protein bands comigrated with the overexpressed protein band, which has the same apparent mobility as the purified cFos. To determine whether those

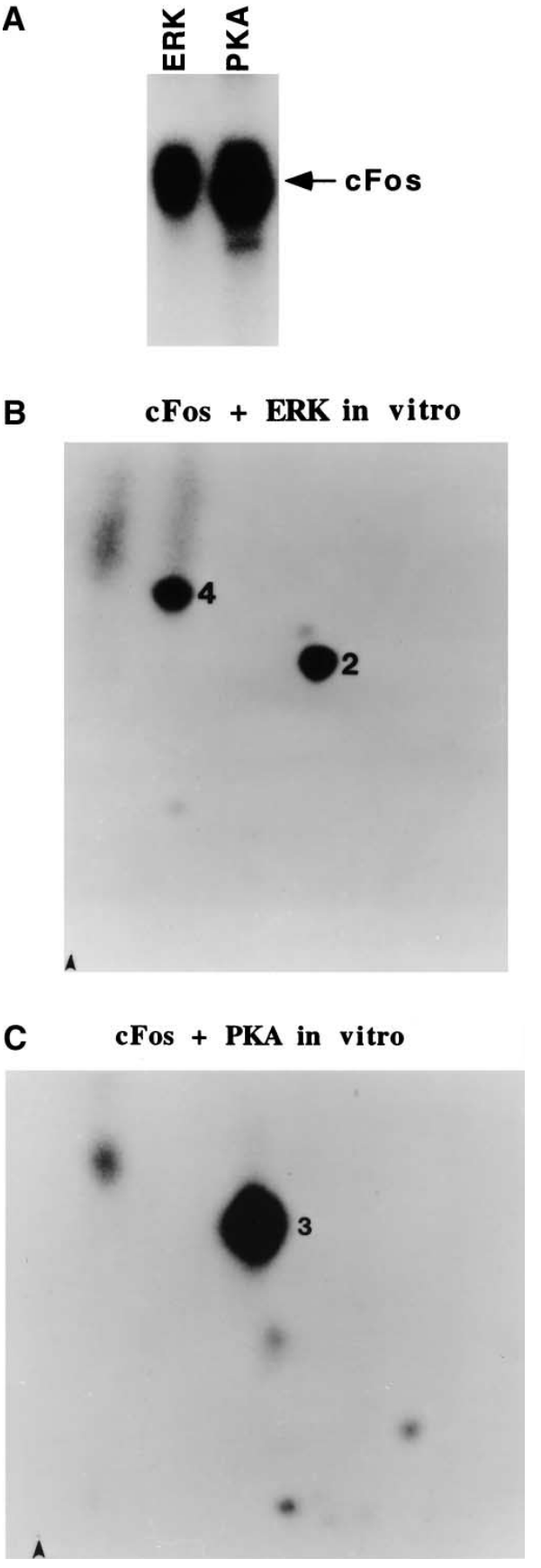

Fig. 4. Phosphorylation of c-Fos by ERK1/2 and PKA in vitro. (A) Purified c-Fos was mixed with ERK1/2 or PKA as indicated in kinase buffer containing [γ-^{32}P]ATP. Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. Bands corresponding to c-Fos are indicated. (B) Two-dimensional tryptic map of c-Fos phosphorylated in vitro by ERK1/2. (C) Two-dimensional tryptic map of c-Fos phosphorylated in vitro by PKA. 2, 3 and 4 refer to the principal phosphopeptides that migrate like phosphopeptides derived from the C-terminal region of c-Fos as described by Tratner et al. [14].

faint bands which comigrated with purified cFos represent low level expression of cFos, a Western blot analysis was performed using a cFos N-terminal specific monoclonal antibody ^{14}Cl [9]. As shown in Fig. 2B, those faint bands did not contain cFos proteins and the overexpressed band reacted with the antibody strongly. Furthermore, the result demonstrated that the simple optimization of ten rare arginine codons can improve cFos expression in bacteria from the undetectable level to about 20% of total bacterial proteins.

To test whether the purified c-Fos was biologically active, gel-retardation was used to examine the interaction of c-Fos with a TRE probe and its interaction with CB4, a cJun/Jun B chimera containing the N-terminal region of cJun and the C-terminal DNA-binding region of JunB [8]. As expected, CB4 did not bind TRE efficiently while cFos did not at all (Fig. 3). However, cFos stimulated CB4-TRE interaction significantly (Fig. 3), suggesting that the bacterially-expressed and purified c-Fos was able to interact with CB4 to form a CB4·cFos complex to bind TRE efficiently, a property expected of biologically active c-Fos proteins. Interestingly, the CB4·cFos-TRE complex seems to migrate slightly faster than the CB4-TRE complex. A likely explanation is that the CB4·cFos-TRE is a more compact complex than the CB4.CB4-TRE is and thus migrates faster.

c-Fos was previously shown to contain ERK (extracellular signal regulated kinase) and PKA (protein kinase A) phosphorylation sites [13,14]. To further test whether the purified c-Fos was biologically active, the purified c-Fos was incubated with ERK1/2 and PKA, respectively, in the presence of γ-^{32}P-ATP. The purified c-Fos was efficiently phosphorylated by both ERK1/2 and PKA (Fig. 4A). To ascertain that ERK1/2 and PKA phosphorylated the purified c-Fos at the appropriate sites, two-dimensional (2D) tryptic phosphopeptide mapping was performed with the c-Fos proteins phosphorylated by ERK1/2 and PKA. The 2D map for c-Fos phosphorylated by ERK1/2 contained two spots, spot 2 and spot 4 (Fig. 4B). The 2D map for c-Fos phosphorylated by PKA contained one predominant spot, spot 3 (Fig. 4C). The results are consistent with the earlier results that ERK1/2 is able to phosphorylate c-Fos at Ser 374 to give rise to the spot 2 and spot 4 [10] and PKA is able to phosphorylate c-Fos at Ser 362 to give rise to the spot 3 [14].

4. Discussion

In this report, I describe the high-level expression of mouse c-Fos in *E. coli* and purification of biologically active c-Fos from the over-producing strain. It has been known for some time that there are significant differences of codon usage between different species [2]. This is especially true between the prokaryotic and eukaryotic species [12]. This difference has been used to explain the difficulty encountered when trying to express certain mammalian proteins in *E. coli*.

Earlier studies had focused either on achieving high levels of expression by using synthetic cDNAs with optimal codons or on studying the effect of rare codons by inserting them into otherwise highly expressed cDNAs in bacteria. Here, I offer a clear example that codon usage indeed affects expression of mammalian genes in *E. coli* dramatically, from undetectable to 20% of total bacterial proteins. Surprisingly, although there is a fairly large number of unfavorable codons in c-fos sequence, optimization of ten arginine codons clustered around the DNA binding domain of c-Fos is all that is necessary for high-level expression of mouse c-Fos in *E. coli*. This observation clearly has implication for expressing other members of fos family in *E. coli* and probably for expressing many other mammalian genes in *E. coli* as well. The easy availability of c-Fos and other mammalian proteins from *E. coli* will clearly have some advantages over other systems in certain applications and will therefore help researchers study the proteins of their interests.

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